

HERMON-TAYLOR et al
Appl. No. 09/646,568
Atty. Ref.: 117-319
Amendment
September 15, 2011

AMENDMENTS TO THE SPECIFICATION:

Please amend the specification as follows:

Please replace the paragraph spanning line 7 on page 45 through line 14 on page 46 with the following replacement paragraph:

Mpa specific primers are designed with concatenated Sad sites in p12 and a PstI site in p13 at the 3' ends (P12:AGCGAGCTCACGTGACTGAAGCC (SEQ ID NO:6) ; P13:GCTCTGCAG CCGAACACAAACGC (SEQ ID NO:7)). A 1371 bp PCR product is amplified, purified by gel electrophoresis and cleaned with Qiagen gel clean column. The product is then cloned into T-Vector (Promega) and transformed into *E.coli*. The plasmid is grown up, purified and a PstI insert fragment removed and purified as before. This is subcloned into a mycobacterial shuttle vector. This vector contains a mycobacterial ori, *E.coli* ori, hygromycin antibiotic marker and the hsp60 promoter immediately upstream of the insertion locus. The resulting construct is transformed into *E.coli* and purified to 1mg/ml. This vector is then sequenced to check that the PCR step and cloning steps do not introduce errors into the sequence and that the sequence is inserted in the correct orientation in the vector. *M.smegmatis* (strain MC²155) is grown into exponential phase, washed twice in 10% glycerol and diluted to OD260:0.5 in TE x1 (Tris 10mM EDTA 0.1mM). 1µg of plasmid is added and the mixture pulsed at 1000Ω:2.5kV:25fD using a BioRad electroporation unit. Cells are recovered in 500µl SOC (2g Tryptone, 0.5g Yeast extract, 1ml 1M NaCl, 0.25ml 1MKCl, 1ml Mg Salts, 1ml 2M Glucose) at 37°C for 3 hours and then plated onto Middlebrooks 7H11 : 45µg/ml

Hygromycin selection plates. Transformants are selected and *mpa* presence checked by *mpa* specific P12/P13 PCR. Expression of *mpa* is also checked by making total mRNA preps of transformants in exponential growth phase. This is done by pelleting a 4 day 30ml culture (in Middlebrooks 7H11 : 45µg/ml Hygromycin broth) at 3,000 xg for 20 minutes. This is resuspended in 200µl RNAse free water and transferred to a ribolyser tube containing silica beads. 500µl of DSA solution (Divolab No.1:9.6ml, 500mM Na Acetate(pH4.0) 24ml, RNAse free water 66.4ml) 500µl Acid Phenol (Water saturated phenol with Na Acetate at pH4.0) and 100µl chloroform/isoamyl alcohol (24: 1). This is then ribolysed at a speed setting of 6.5 for 45secs. The tubes are then microfuged for 10 minutes at 13,000 xg. Total mRNA is extracted with equal volume of chloroform/isoamyl alcohol and then precipitated at -70°C with isopropanol for 2 hours. This is then microfuged at 13,000 xg for 20 minutes dried and resuspended in RNAase free water. Samples are treated with DNAase and then cDNA produced using the P12 primer as template with SuperscriptII Reverse Transcriptase (GibcoBRL). PCR using P12/P13 primers is then performed showing bands of the correct size. Controls without DNAase and with RNAase H treatment are performed in parallel. This demonstrates transcribed copies of *mpa* present in *M.smegmatis*. Translation is revealed by taking exponential growth cultures of transformants and whole cell lysates using 1 minute sonication in 2% SDS-PAGE buffer. These whole cell protein extracts are then electrophoresed on 1% polyacrylamide gels and western blotted onto nylon membranes. These are then hybridised with rabbit raised, *mpa* peptide antisera and developed with anti-rabbit HRP conjugate/ECL peroxide system.

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Please insert the attached Sequence Listing in place of any previously-filed version of the Sequence Listing.